PROTEOLYSIS OF PUROMYCIN-PEPTIDES IN RABBIT RETICULOCYTES: DETECTION OF A HIGH MOLECULAR WEIGHT OLIGOPEPTIDE PROTEOLYTIC SUBSTRATE

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1. Introduction

Many types of cells (*Escherichia coli*, HeLa, human fibroblast, rat hepatoma, and the reticulocyte of the rat, mouse and rabbit) have the capability to degrade selectively proteins whose structures deviate significantly from that of normal gene products; such proteins are termed abnormal [1]. The mechanism of this selective proteolysis is unclear although it may be energy-dependent [2,3]. In the present study we have induced the synthesis of labelled protein of shortened chain length in rabbit reticulocytes by incubation with the antibiotic puromycin, a chain terminator, and monitored, by gel filtration, subsequent changes in protein size. We show that puromycin promotes the synthesis of high MW (over 100 000) peptide aggregates which rapidly disappear after removal of the antibiotic: we suggest that these peptide complexes may be proteolytic substrates in the degradative process.

2. Materials and methods

2.1. *Chemicals*

L-[U-^{14}C]leucine (330 mCi/mmol) was obtained from the Radiochemical Centre (Amersham) and puromycin dihydrochloride from the Sigma Chemical Company. Radioactive samples were counted, using PCS as a scintillant (Hopkin & Williams), in a Packard liquid scintillation spectrophotometer.

2.2. *Preparation, incubation and labelling of reticulocytes*

Reticulocytes were prepared from male New Zealand white rabbits by the method of Lingrel and Borsook [4] with the modifications of Denton and Arnstein [5]. Cells were resuspended in the standard medium of Lingrel and Borsook [4], with the omission of the amino acid mix, and incubated at 37°C for 10 min in a shaking water bath. The amino acid mix [4], [^{14}C]leucine (0.65 μCi/ml final concentration) and puromycin dihydrochloride (either 5 or 25 μg/ml) were then added and incubation continued for 5 min.

2.3. *Determination of protein degradation*

Reticulocytes were 'pulse-labelled' with [^{14}C]-leucine for 5 min as above, washed twice with saline [4] containing 50 mM [^{12}C]leucine, and centrifuged at 600 × *g* for 10 min. The cells were resuspended in standard medium [4] that had been preheated to 37°C and contained 20 mM [^{12}C]leucine. 25 μl samples were removed immediately (zero time sample) and after 60 min incubation, precipitated with 2 ml 5% trichloroacetic acid (w/v) left overnight at 4°C and filtered on Whatman GFC glass fibre discs. 1 ml of filtrate was added to 2 ml PCS scintillant and acid-soluble radioactivity determined. Total radioactivity was determined by bleaching a 25 μl sample (1 ml 0.5 M NaOH plus 2 drops of 20 vol. H_2O_2 and incubated at room temperature overnight) which was counted as above. Protein breakdown is expressed as the increase of acid-soluble radioactivity as a percentage of that originally in protein (acid-precipitable fraction).

2.4. *Preparation of cell-free lysates*

Packed cells were lysed 1:1 (v/v) with water on ice for 10 min, and then centrifuged for 10 min at 600 × *g* to remove cell debris.

2.5. *Gel filtration*

Cell-free extracts (prepared as above) together with the initial supernatant plasma fraction (because it contained the majority of the free [^{14}C]leucine) were applied to a column (350 × 14 mm) of Sephadex G100 (Pharmacia) and eluted with 0.1 M Tris–HCl (pH 7.6) unless stated to the contrary. 0.5 ml of each fraction (2.5 ml) was bleached (if necessary) and the total radioactivity determined.

2.6. *Sodium dodecyl sulphate polyacrylamide gel electrophoresis*

The method employed was based on that of Weber and Osborn [6] and carried out in 115 × 8 mm tubes. 17.5% Acrylamide gels were made by mixing 14.5 ml acrylamide solution (35 g acrylamide and 0.66 g methylenebisacrylamide per 100 ml), 15.0 ml gel buffer containing 8.7 g $NaH_2PO_4.2H_2O$, 19.9 g Na_2HPO_4 and 2.0 g of sodium dodecyl sulphate (SDS), 45 μl *NNN'N'*-tetramethylethylene and 0.75 ml ammonium persulphate (15 mg/ml). The electrophoresis buffer was made by diluting the gel buffer 1:1 with distilled water. Samples for electrophoresis were boiled for 10 min after the addition of an equal volume of a solution containing 1.1 ml β-mercaptoethanol, 0.2 g SDS, 12.6 g glycerol made up to 50 ml with electrophoresis buffer. 100 μl of sample and 15 μl bromophenol blue were applied to each gel and electrophoresis carried out at a constant current (6 mA/gel) for 20 h at room temperature. Gels were sliced into 2 mm sections, soaked overnight in 0.5 ml electrophoresis buffer and the radioactivity determined after addition of 1 ml of PCS scintillation fluid.

Table 1

Proteolysis of protein synthesized in rabbit reticulocytes pulse-labelled with [^{14}C]leucine in the presence of 5 and 25 μg/ml puromycin dihydrochloride

Puromycin dihydrochloride concentration (μg/ml)	% Proteolysis in 60 min
0	2.5 (12 412)[a]
5	39.6 (7861)
25	82.5 (4789)

[a] Figures in parentheses denote the total acid precipitable cpm detected in a 25 μl sample of the incubation mixture

3. Results

Pulse-labelled reticulocyte protein synthesized in the presence of 5 μg/ml puromycin dihydrochloride was rapidly degraded (table 1 and [7,8]) and incuba-

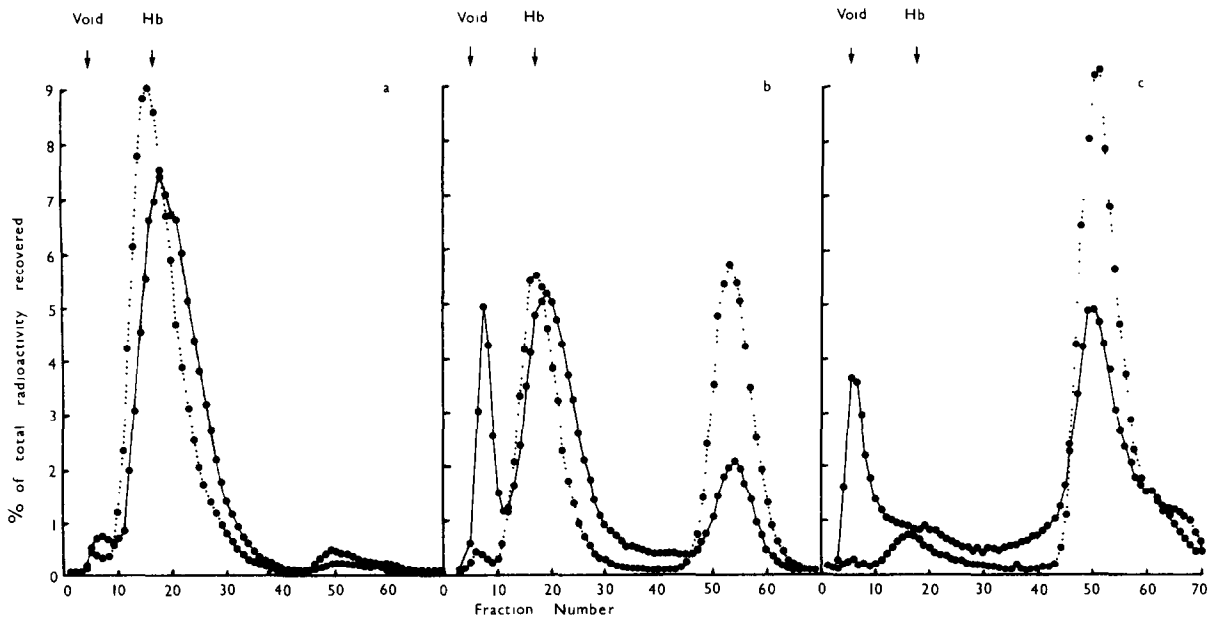

Fig.1. Proteolysis in rabbit reticulocytes: Sephadex G100 chromatography of extracts of cells labelled with [^{14}C]leucine in the presence of puromycin (see Section 2). (a) Control cells; (b) cells labelled in the presence of 5 μg/ml puromycin dihydrochloride; (c) cells labelled in the presence of 25 μg/ml puromycin dihydrochloride. Period of proteolysis: o——o, 0 min; o···o, 60 min.

tion with 25 μg/ml puromycin dihydrochloride resulted in greater proteolysis (table 1).

Gel filtration of the 600 × *g* supernatant fractions of lysates of the 5 μg/ml puromycin treated cells revealed the presence of high molecular weight radioactive material in or close to the void volume (fig.1b) in the pulse-labelled cells. Subsequent incubation in the absence of the antibiotic and in the presence of excess [^{12}C]leucine resulted in the disappearance of this material (fig.1b). Labelling in the presence of 25 μg/ml puromycin generated a larger proportion of the radioactive void volume material which also disappeared during the 'chase period' (fig.1c). Control cells showed low levels of proteolysis (table 1) and contained only a small amount of this material (fig.1a) which also disappeared during the 'chase'. Similar analyses were performed on a number of separate experiments using puromycin at 5 or 25 μg/ml. Fig.2 shows that proteolysis was consistently related to that proportion of the total acid-precipitable radioactivity which eluted in the void volume region.

We considered whether the radioactive void volume material was ribosome-associated. Sedimentation of the lysate at 100 000 × *g* did not greatly diminish the amount of radioactivity which eluted in the void volume of the Sephadex columns and sedimentation on sucrose density gradients revealed that the material did not migrate with either ribosomes or ribosomal

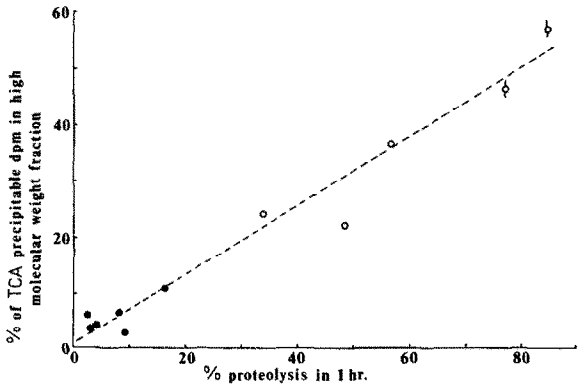

Fig.2. Proteolysis of puromycyl-peptides in rabbit reticulocytes: relationship between percentage proteolysis in whole cells and the proportion of total acid precipitable radioactivity in the 600 × *g* supernatant of extracts of [^{14}C]-leucine pulse-labelled cells which eluted in the void volume region (high MW fraction) of a Sephadex G100 column. Cells pulse-labelled in presence of 25 (ϕ), 5 (○) and 0 (●) μg/ml puromycin dihydrochloride.

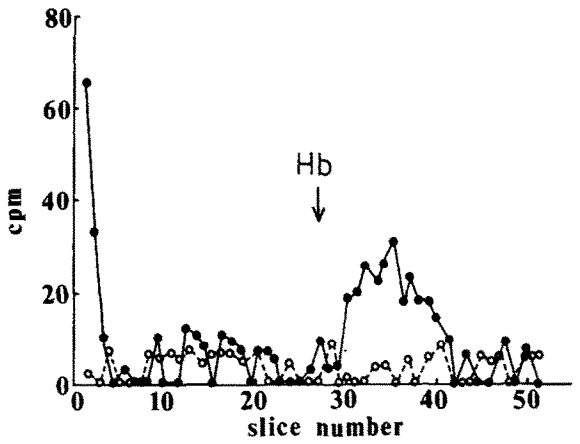

Fig.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the [^{14}C]leucine-labelled high MW fraction (void volume material from a Sephadex G100 column) prepared from rabbit reticulocytes treated with 25 μg/ml puromycin dihydrochloride. Cell extracts of [^{14}C]leucine-labelled cells were prepared, centrifuged at 100 000 × *g* for 2 h, and the radioactive void volume material which eluted with 0.1 M NH_4HCO_3 from a Sephadex G100 column was collected, lyophilised and separated by electrophoresis as described in Section 2. Period of proteolysis in original cells: •, 0 min; ○, 60 min.

subunits (results not shown). These results suggest that the labelled void volume material was not ribosome-associated.

Elution of the Sephadex G100 columns with 0.1 M NH_4HCO_3 (pH 9) which gave profiles virtually identical to those in fig.1 (not shown) allowed the concentration of the void volume material by lyophilization. SDS polyacrylamide gel electrophoresis revealed that the void volume material of cells pulse-labelled in the presence of 25 μg puromycin/ml was composed primarily of peptides of 6000–12 000 MW (fig.3), which disappeared during the chase period (fig.3). In addition some very high MW protein was present remaining at the origin (which also disappeared during the chase), the nature of which is unknown.

4. Discussion

It is accepted that puromycin brings about the release from ribosomes of polypeptides of shortened chain length. It was therefore surprising to detect large amounts of [^{14}C]leucine-labelled material of MW greater than 100 000 in extracts of puromycin-treated

cells (figs.1b and 1c). The kinetic behaviour of this high MW material (fig.1) and its increased abundance when high levels of proteolysis occurred (fig.2) are consistent with it being a degradative substrate. Electrophoresis under denaturing conditions revealed (fig.3) this material to be composed mostly of peptides of molecular weight less than those of the complete globin chains. Pulse-labelling reticulocytes with [^{3}H]puromycin also resulted in the appearance of radioactivity in the void volume region of a Sephadex G100 column elute which subsequently disappeared during the chase period (not shown). Thus it can be argued that the shortened peptides, at least some of which are still attached to puromycin, form an oligopeptide high molecular weight complex which is subsequently subject to proteolytic attack.

In addition to the puromycin-induced pulse-labelled high molecular weight material, fig.1 has other features worthy of comment. First, during the chase period there is in figs.1a and b a loss of material from the trailing edge of the haemoglobin peak, together with a small decrease in the elution volume of this major peak. This we take to result from the association of newly synthesized haemoglobin monomers into dimers (note that haemoglobin elutes as a dimer on Sephadex columns). Experiments with Biogel P150 which gave much improved resolution, clearly demonstrated the disappearance of a monomer peak during the chase period. Secondly, puromycin induced an increased amount of material which eluted in fractions 50–60 (fig.1) and which increased further during the chase period. This we take to be the products of catabolism the puromycin peptides to labelled leucine during the 5 min pulse period: alternately such material could represent unaggregated very low MW peptides.

A number of questions may be asked concerning the high MW oligopeptide aggregate and its proteolysis. (i) Is aggregation a necessary prerequisite to the proteolysis of puromycin peptides? (ii) Do the peptides in the aggregate represent the complete puromycin peptides as released from the ribosomes or are they intermediates formed after an initial proteolytic attack? (iii) Is aggregation physiological or merely physical? It may be of interest to note that the products of cyanogen bromide cleavage of denatured *E. coli* alkaline phosphatase can spontaneously aggregate; some can be degraded in cell-free extracts [9]. Aggregation and proteolysis of puromycin peptides has also been described in *E. coli* [3]. Preliminary experiments suggest that aggregated puromycin peptides synthesized in the reticulocyte can also be degraded in cell-free preparations by a process which may be stimulated by ATP (R. S. Daniels, E. M. Atkinson and A. R. Hipkiss, unpublished observations). Mature reticulocytes defective in their ability to degrade puromycin-peptides [10–12] can produce the high MW aggregate but appear to possess a decreased ability to catabolise it (M. J. McKay and A. R. Hipkiss, unpublished observations).

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